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Application of Box-Behnken Design for Optimization of Poly- γ -Glutamic Acid Production by *Bacillus licheniformis* SAB-26

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Abstract: Optimization of poly- γ -glutamic acid (PGA) produced by the glutamate-independent *Bacillus licheniformis* SAB-26 has been carried out using response surface methodology. Predicted maximum PGA yield (57.9 g L^{-1}) was calculated using non-linear optimization algorithm powered by Microsoft Excel, where the optimized medium composition was (g L^{-1}): casein hydrolysate, 12.2; ammonium sulfate, 20; K_2HPO_4 , 27 and KH_2PO_4 , 27 after 72 h. A verification experiment was carried out to compare predicted and experimental results, where experimental yield reached (59.9 g L^{-1}) in a good sign of model validity. Pulsed feeding experiment of citrate at different time intervals revealed increasing the polymer production to 88 g L^{-1} .

Key words: *Bacillus licheniformis*, Box-Behnken experimental design, poly- γ -glutamic acid optimization

INTRODUCTION

Poly- γ -glutamic acid (γ -PGA) is an unusual anionic naturally occurring homo-polyamide made of D- and L-glutamic acid monomers, which are connected by amide-linkage between α -amino and γ -carboxyl groups (Thorne *et al.*, 1954). The potential applications of γ -PGA as thickener, humectant, bitterness relieving agent, sustained release material and drug carrier have been of interest in the last decade.

γ -PGA has been produced by several *Bacillus* species in the medium as a product of fermentation. Poly glutamate producing bacilli were divided into two groups: one requires the addition of glutamic acid to the medium to stimulate the γ -PGA production and cell growth and called glutamate-dependent. The other is capable of producing γ -PGA via *de novo* pathway in the absence of glutamic acid and called glutamate-independent. The later group most notably are *B. subtilis* TAM-4 (Ito *et al.*, 1996), *B. subtilis* C1 (Shih *et al.*, 2005) and *B. licheniformis* SAB-26 (Soliman *et al.*, 2005).

In a previous study we were able to identify the most significant medium components affecting the production of γ -PGA by the glutamate-independent *B. licheniformis* SAB-26 using Plackett-Burman experimental design (Soliman *et al.*, 2005). In order to approach the optimum level of γ -PGA production, a second optimization experiments should be carried out. In the present work we took the advantages of statistical designs to implement Response Surface Methodology (RSM), which has been

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applied to optimize various microbial metabolites (Abdel-Fattah *et al.*, 2005; Abdel-Fattah *et al.*, 2007), in order to optimize the key medium parameters using non-linear optimization algorithm. Furthermore, for the purpose of improving production, the effect of pulsed feeding of citrate at different intervals was investigated in cultures of *B. licheniformis* SAB-26.

MATERIALS AND METHODS

The present study was carried out in laboratories of Bioprocess development Department, Genetic Engineering and Biotechnology Research Institute, (Alexandria) during the period March-June 2006.

Microorganism

Bacillus licheniformis SAB-26, isolated from waste material deposited from (El-Nasar Company for Tanning of Leather, Alexandria, Egypt) by our laboratory (Soliman *et al.* 2005), was maintained on nutrient agar slants composed of (g L⁻¹): peptone; 5, beef extract; 3, NaCl; 2 and agar; 20. Stock culture was sub-cultured at regular intervals of one month and stored under refrigeration.

Growth and Production Conditions

The bacterium was allowed to grow in 50 mL aliquot of nutrient broth dispensed in 250 mL Erlenmeyer flask and incubated at 37°C for 12 h at (200 rpm). One percent inoculum of the overnight culture was used to inoculate the preoptimized basal production medium, formulated from previous study (Soliman *et al.*, 2005), of the following composition g L⁻¹: glucose, 5; glycerol, 10; citrate, 12; casein hydrolysate, 8; (NH₄)₂SO₄, 16; K₂HPO₄, 20; KH₂PO₄, 20; MgSO₄, 0.55; MnSO₄, 0.3; FeSO₄, 0.03; CaCl₂, 0.3 and ZnCl₂, 0.03 at pH 6 and 37°C. The γ -PGA was determined in culture supernatants after clarifying cultures by precipitation method. For the recovery of poly glutamic acid, the most common technique was applied (Ashiuchi *et al.*, 1999), where the fermentation broth was first centrifuged to remove cells (30 min, 5000 rpm at 4°C). The poly glutamic acid in the supernatant was precipitated using ice-cold ethanol (1:2 volume ratio), then kept at 4°C overnight. The resulting crude precipitated poly glutamic acid was separated by centrifugation (45 min, 5000 rpm, 4°C). For further purification, the precipitation step was repeated. Finally the precipitate was overnight dried at 60°C and weighed.

Response Surface Experimental Design

Based on the results obtained previously (Soliman *et al.*, 2005), the Box-Behnken experimental design (BBD) based on response surface methodology was conducted to locate the true optimum concentrations of casein hydrolysate, ammonium sulfate, K₂HPO₄ and KH₂PO₄ for γ -PGA production. For these four variables, a design matrix based on 27 trials involving three center points was constructed. The levels of each factor are given in Table 1. The experimental results of the RSM were fitted with the second-order polynomial equation by the multiple regression technique.

$$Y = B_0 + \sum_{i=1}^k B_i x_i + \sum_{i=1}^k B_{ii} x_i^2 + \sum_{i < j} B_{ij} x_i x_j \quad (1)$$

Table 1: Variables and their levels (g L⁻¹) employed in Box-Behnken experiment for optimization of culture conditions affecting PGA production by *B. licheniformis* SAB-26

Variables	-1	0	1
Casein hydrolysate (X ₁)	7	10.0	13
(NH ₄) ₂ SO ₄ (X ₂)	15	17.5	20
K ₂ HPO ₄ (X ₃)	19	23.0	27
KH ₂ PO ₄ (X ₄)	19	23.0	27

Where Y is the predicted response, B_0 , B_i , B_{ii} , B_{ij} are constant coefficients, and x_i and x_j are the coded independent variables or factors. The quality of fit of the second-order model equation was expressed by the coefficient of determination R^2 , and its statistical significance was determined by F-test. The significance of the regression coefficients was tested by a t-test.

Fed-batch Pulsed-feeding Experiment

In order to study the effect of pulsed feeding of tri-sodium citrate at different intervals, a final concentration of citrate (12 g L^{-1}) were added on two, three or four pulses after 12, 8 and 6 h intervals, respectively.

RESULTS AND DISCUSSION

Optimization of Significant Medium Components for γ -PGA Production by *B. licheniformis* SAB-26

In order to bring about the optimum culture conditions for production of γ -PGA, a previous screening study has been performed to evaluate the efficacy of different medium and culture variables on the γ -PGA production from *B. licheniformis* SAB-26 (Soliman *et al.*, 2005). It has been shown that *B. licheniformis* SAB-26 is a glutamate-independent bacteria producing poly glutamate in the absence of glutamic acid. A pre-optimized basal production medium formulated from the previous study and described in the materials and methods section showed a production of 46.5 g L^{-1} γ -PGA in shaking flask conditions. Previously, (Cromwick *et al.*, 1996; Yoon *et al.*, 2000) reported that under optimized conditions *B. licheniformis* ATCC9945a can produce the polymer in amounts ranging from 20 to 50 g L^{-1} of the culture fluid.

In our previous study, we showed negative effect of glucose and glycerol on γ -PGA production (Soliman *et al.*, 2005). In order to simplify the production medium for better yield, glucose and glycerol were omitted from medium composition that resulted in increasing PGA yield to reach 52.4 g L^{-1} . As a result, the formulated pre-optimized medium in this recipe could be considered as a basal condition to start optimizing the most significant variables.

In order to find out the combination that brings maximum PGA yield by *B. licheniformis* SAB-26, the most positive significant medium components, namely casein hydrolysate, $(\text{NH}_4)_2\text{SO}_4$, K_2HPO_4 , and KH_2PO_4 were studied.

To explore the sub region of the response surface in greater detail, a second-order model or higher is required to approximate the response (Fig. 1). The full quadratic model (Eq. 1) was most frequently used to approximate the high-order response and was adequate in most cases (Mason and Gunst, 1989; Montgomery, 1991). In the present study, Box-Behnken Design (BBD) was applied to fit a full quadratic equation and its experimental results together with predicted values from the model Eq. 1.

Second-order Model Equation

By applying multiple regression analysis on the experimental data shown in Table 2, the experimental results of the BBD were fitted with the polynomial Eq. 1, and the second-order polynomial equation obtained for γ -PGA production is shown in Eq. 2.

$$Y_{(\text{PGA}, \text{g L}^{-1})} = 49.97 + 1.005 X_1 + 3.963 X_2 + 3.773 X_3 + 2.048 X_4 + 0.43 X_1 X_2 + 0.495 X_1 X_3 - 0.08 X_1 X_4 - 0.195 X_2 X_3 - 2.588 X_2 X_4 - 1.855 X_3 X_4 - 1.272 X_1^2 - 0.161 X_2^2 - 0.857 X_3^2 + 3.169 X_4^2 \quad (2)$$

The fit of model was checked by the R^2 , which was calculated to be 0.8975, indicating that 89.75% of the variability in the response could be explained by the model. The significant variables

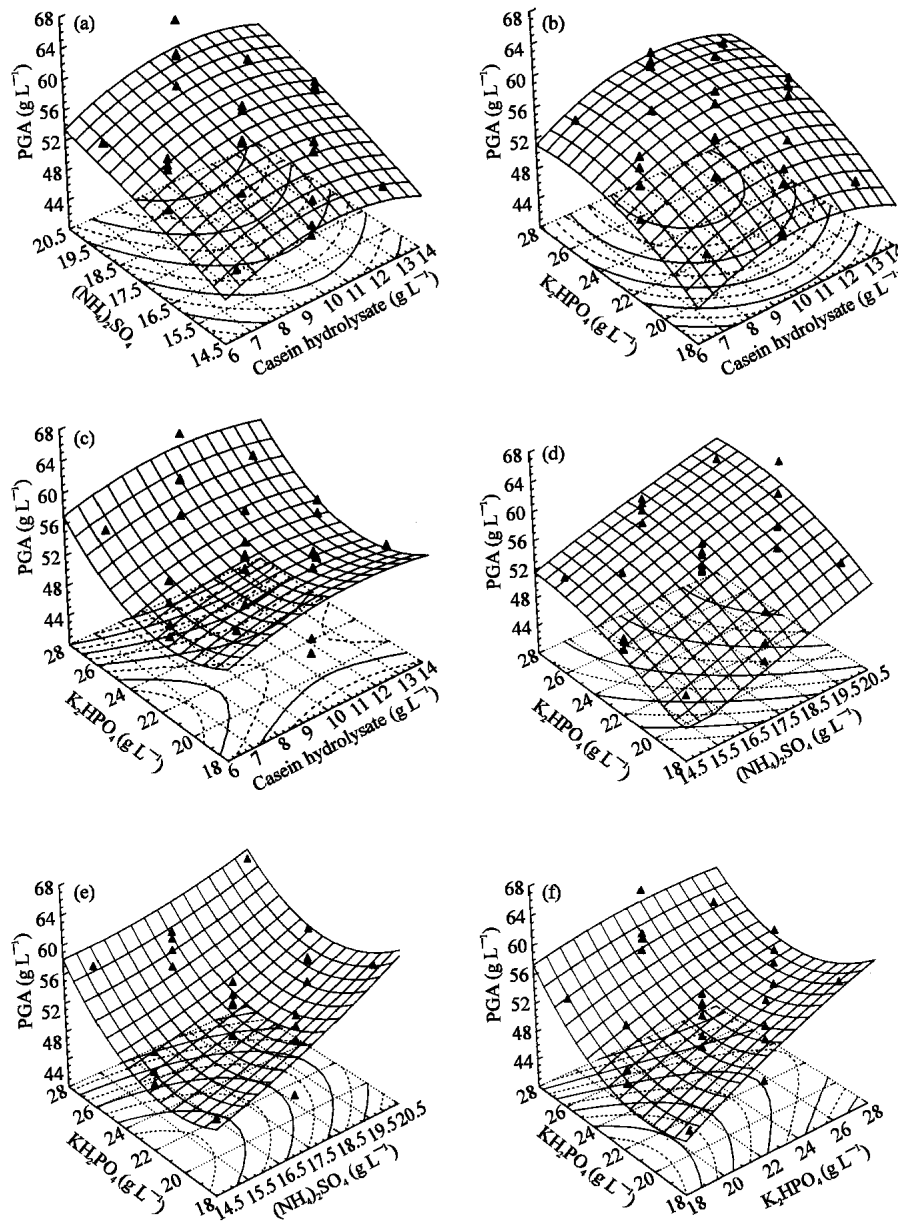


Fig. 1(a-f): Response surface plots for the yield of PGA (g L^{-1}) from *B. licheniformis* SAB-26 as affected by culture conditions

can be identified by means of their Student t-test values. The results revealed that the first-order term of $(\text{NH}_4)_2\text{SO}_4$, K_2HPO_4 and KH_2PO_4 displayed significant effect on the γ -PGA yield at a 5% level. In addition, interaction term of $(\text{NH}_4)_2\text{SO}_4$ - KH_2PO_4 as well as quadratic effect of KH_2PO_4 presented significant effects on the γ -PGA yield at a 5% level. However, all the other terms presented insignificant effects on the γ -PGA yield at a 5% level. Nevertheless, these terms were not omitted from the model equation since its adequate fit could be confirmed. The test statistics F-values for the overall

Table 2: Box-Behnken factorial experimental design, representing the response of PGA production as influenced by casein hydrolysate, $(\text{NH}_4)_2\text{SO}_4$, K_2HPO_4 and KH_2PO_4

Trial #	Casein hydrolysate (X_1)	$(\text{NH}_4)_2\text{SO}_4$ (X_2)	K_2HPO_4 (X_3)	KH_2PO_4 (X_4)	PGA (g L^{-1})
1	10	15.0	19	23	42.34
2	10	20.0	27	23	57.02
3	10	15.0	23	27	55.46
4	10	20.0	19	23	52.16
5	13	17.5	23	19	53.14
6	10	17.5	27	19	54.89
7	10	20.0	23	19	57.32
8	10	20.0	23	27	62.60
9	7	17.5	27	23	51.54
10	13	17.5	27	23	53.70
11	7	17.5	23	19	50.90
12	13	20.0	23	23	51.70
13	10	17.5	23	23	49.80
14	10	17.5	27	27	55.64
15	7	15.0	23	23	42.72
16	7	17.5	19	23	44.62
17	7	17.5	23	27	52.60
18	10	15.0	27	23	47.98
19	10	17.5	23	23	50.20
20	13	17.5	23	27	54.52
21	10	17.5	19	19	41.70
22	10	17.5	19	27	49.87
23	13	17.5	19	23	44.80
24	10	15.0	23	19	44.00
25	13	15.0	23	23	44.64
26	10	17.5	23	23	49.90
27	7	20.0	23	23	48.06

regression is significant at the upper 5% level and the lack of fit is insignificant. These facts further supported that the second-order model is very adequate in approximating the response surface of the experimental design.

On analysing Eq. 2 with Solver, based on non-linear optimization algorithm, maximum PGA production was found to be 57.9 g L^{-1} when concentrations of tested variables were (g L^{-1}): casein hydrolysate, 12.2; ammonium sulfate, 20; K_2HPO_4 , 27 and KH_2PO_4 , 27. Verification of the calculated optimum was done with a culture medium representing this optimal point and yielding $59.9 \pm 2.3 \text{ g L}^{-1}$ (average of three repeats). The excellent correlation between predicted and experimental values justifies the validity of the response model and the existence of an optimum point.

This study showed that γ -PGA production by *B. licheniformis* SAB-26 was more than 5 folds increased from 11.2 to 59.9 g L^{-1} when the strain was cultivated in the optimal medium developed by BBD, as compared to basal medium used in the previous study (Soliman *et al.*, 2005). Therefore, the BBD proved to be a powerful and useful tool for enhancing γ -PGA production. In comparison, the γ -PGA yield obtained by *B. licheniformis* SAB-26 was 3 folds as the yield obtained by the glutamate dependent *B. licheniformis* CCRC 12826 in a medium optimized through central composite design CCD (Shih *et al.*, 2002). The same group were able to improve the PGA yield by optimizing the concentration of ammonium chloride, which is supposed to be similar to ammonium sulfate, where a maximum yield of 33.2 g L^{-1} was obtained (Lu *et al.*, 2004).

Fed-batch Pulsed-feeding Experiment

Since PGA is an extracellular polymer having high molecular mass, the culture medium becomes highly viscous upon its production. The increased viscosity is likely to decrease the volumetric O_2

transfer, and leads to O_2 limitation. This problem becomes more significant in fed-batch culture, in which cells need to be grown to a higher density (Lee, 1996). Giannos *et al.* (1990) reported that 39 g L^{-1} could be produced in 5 days by adding feeding solution after 3 days of fermentation. The productivity was about $0.33 \text{ g L}^{-1} \text{ h}^{-1}$, which was rather low. For the commercial application of PGA in large amounts, it is necessary to enhance the productivity. Yoon *et al.* (2000) have reported a simple strategy for the production of PGA by pulsed-feeding of citric acid and L-glutamic acid that led to high productivity (35 g L^{-1}) in fed-batch culture of *Bacillus licheniformis*.

In the present study, an overall concentration of tri-sodium citrate (12 g L^{-1}) were added after 12 h of growth in the optimized medium as two, three or four pulses at 12, 8 and 6 h intervals, respectively. Figure 2 illustrates the growth pattern as well as the final volumetric production of PGA under different citrate pulsed feeding regime. On growth level, a remarkable diauxic growth is recognized in the 2-pulse feeding with specific growth rates of 0.11 and 0.045 h^{-1} in the two phases, respectively. A remarkable decrease of the growth rate in the first growth log phase is illustrated to reach 0.077 h^{-1} in both 3 and 4 pulse feeding experiments. On the other hand, a decrease of specific growth rate was measured in the third phase of growth between 3 pulse (0.038 h^{-1}) and 4 pulse (0.027 h^{-1}) feeding experiments. PGA production was correlating to the growth pattern, where maximum volumetric production (89 g L^{-1}) was measured in the 2-pulsed feeding experiment that showed highest growth rate and maximum OD_{600} 5.25. PGA production was significantly decreased in the 4-pulsed feeding experiment (81.5 g L^{-1}), although the maximum measured OD_{600} (5.38) was higher than that measured in the 2-pulsed feeding experiment. So far, there is no publication reported

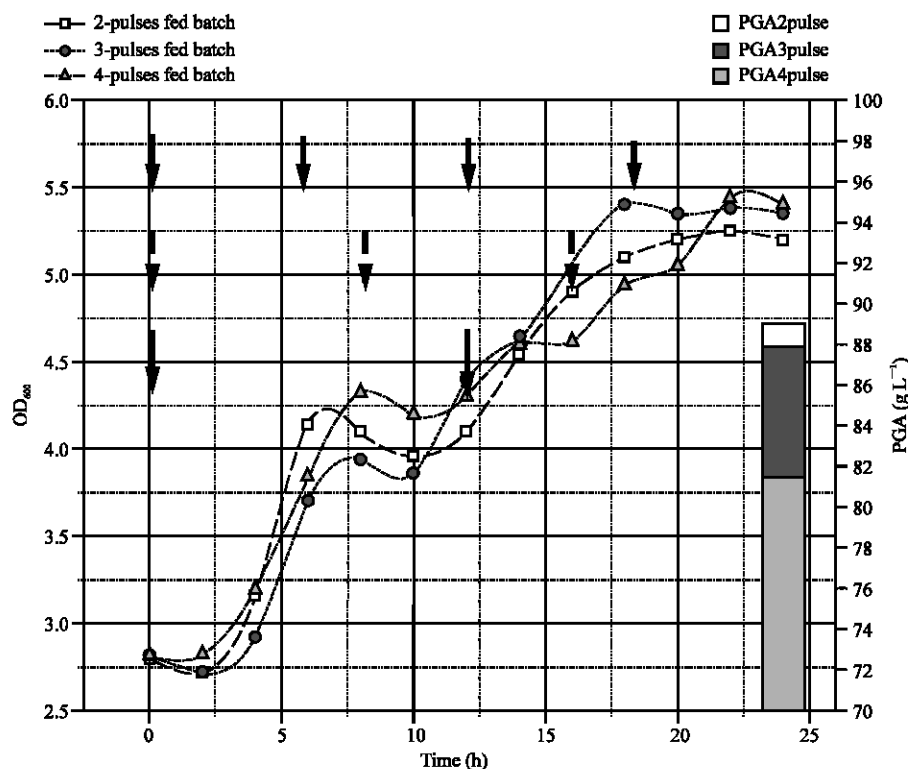


Fig. 2: Growth pattern and overall PGA production in citrate pulsed feeding experiment of *B. licheniformis* SAB-26 as affected by culture conditions. Arrows representing the feeding regime as two, three and four pulses per experiment

the production of such amount of PGA, in a sign of the importance of the producing strain *B. licheniformis* SAB-26 and its suitability to enter the industrial scale for polymer production.

From the experiment it could be concluded that PGA production is to great extent affected by the first feeding regime, as the product is growth-associated. Production of the biopolymer is inversely proportional to the increase of number of feeding pulses.

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